

STUDIES IN THE BIOLOGY OF THE LEECH.¹ VIII.

MODIFICATIONS IN NEUROLOGICAL MICROTECHNIQUE

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During the last two decades of the nineteenth century there were devised several basic methods of neurological microtechnique.² These made possible the study of the minute structure of the nervous system. Many modifications of these and other techniques have been offered until, at the present, there are probably more variations in neurological methods than in any other. This multitude of divergent procedure is based upon necessity.

In the study of nervous tissue there are many variable factors which influence to a marked degree the cytological as well as histological results. A few of the better known variables are functional condition, structural differentiation, and age of the tissue. The taxonomic position of the organism must be considered, and in many instances, species variation taken into account. In addition, there are many imperfectly known factors demanding variations in procedure that can be determined only after extensive experimentation.

The methods designed to assist in the microscopical examination of the nervous system can be divided into two fundamental groups: the one spoken of as cytological, and the other as histological or anatomical. The former refers to those techniques which, by virtue of minute qualitative differentiating properties, make possible a study of the intimate structure of nerve cells, processes, and their supporting tissues. The latter, histological or anatomical, applies more particularly to the minute structure and composition of nervous tissue. In general this latter type of study is concerned with the architectural arrangement of nerve cells, their processes, and their connections with one another.

The interest in chordates, and more particularly in man, has inspired exhaustive research for adequate neurological methods applicable to this group. To a large extent the methods presented for the study in invertebrate nervous systems have been borrowed, and modified from vertebrate procedures. Exceptions to this were the contributions to invertebrate neurological microtechnique made by some of the early investigators, and their students.

Retzius, Cajal, and deCastro, Smallwood, Rogers, Ascoli, Sanchez, to mention only a few, have each presented numerous modifications in neurological methods. Each has contributed to the growing list of variable procedures employed in the demonstration of nerve elements. Each has presented techniques applicable to the particular field of investigation in which he worked. With all due respect to these and other technicians, problems in invertebrate neurological research have remained unanswered for want of adequate cytological and histological methods.

THE PROBLEM

The behavior of an animal is the result of stimuli acting upon a particular arrangement of anatomical structure under the physiological conditions existing at the time of stimulation. The determination of the histological character of the tissue involved is a necessary prerequisite to an analysis of the causal factors in

¹*Haemopsis marmoratis* (Say).

²These methods are reviewed in Lee, McClung and others.

behavior. The leech, *Haemopsis marmoratis*, (Say) has been used for some time by the writer in studying various aspects of annelid behavior. With the exception of the description of the subepidermal nerve plexus (1933) the authors' reaction studies have, for the most part, been of an analytical nature. Certain conclusions derived from the study of behavior, have, by inference, implied the general pattern of the nervous system. The limited knowledge of the nervous system of this form is to a large extent due to inadequate cytological and histological methods. The writer employing certain well founded principles of neurological procedure presents the following new methods and modifications.

NEUROLOGICAL METHODS

The methods used and the results obtained, as set forth in this paper, pertain specifically to the aforementioned animal. These procedures have not been tried on any other form. To what extent these methods may be applicable to other animals is a matter for further investigation. The discussion which follows should be considered with these points in mind.

RELAXING, KILLING, AND FIXING

These three steps in the preparation of the animal, and more particularly, the tissue to be studied, were considered as one operation. But inasmuch as variations were employed in each of the above, they will be presented separately. These were important details, for in many cases the success of the staining process was dependent upon these as well as subsequent treatments. The appearance of the tissue may be entirely altered by deviations from the prescribed methods.

Relaxing was the first step in the preparation of the leech for subsequent study. The three methods which were used are stated below. Each was employed in accordance with the killing and fixing procedure discussed later in this paper.

RELAXING METHODS

Method I. The leech was placed in a shallow glass dish containing a 0.1% to 0.2% solution of chloretone, to which had been added a pinch of proteolytic enzyme. (Caroid was found satisfactory). This latter, while not absolutely necessary, materially aided in disposing of the copious mucous secreted by the organism. After five minutes in the chloretone solution there was added, drop by drop, a saturated solution of the same, until the leech was completely relaxed. Dissection followed, or if the animal was to be imbedded in toto, it was straightened to normal size in a wax bottom dissecting pan, where it was held in position by pins. The first application of the killing and fixing agent was applied at this time.

Method II. Ether was an equally satisfactory relaxing agent. The leech was placed in a covered dish, into which was previously introduced a wad of cotton saturated with ether. In due time the animal was completely relaxed, and treated as stated in No. I.

Method III. This method differed from Method I or II, only in the anesthetic employed. In this method, alcohol was used in place of chloretone. Animals were prepared in this way when an alcoholic fixing medium was employed.

KILLING AND FIXING METHODS

Killing and Fixing procedure will be considered under one heading. While killing is usually referred to as the act of stopping all life processes and fixing a method of preserving the normal cell structure, the two steps should be accomplished simultaneously. Usually a single medium is employed for this purpose. If the nervous system is dissected prior to final fixation, great care should be exercised in the manipulation of the tissue. The same care should be taken if dissec-

tion is made following killing and fixing. As will be noted later in this paper, the staining methods employed and the results obtained are dependent to a large extent on this step.

Method I. A solution of 10% formalin was used to kill and fix either the dissected tissue, or the entire organism. The usual time of fixation was 24 hours. If no secondary fixing agent was employed following formalin fixation, washing in tap water for 5 minutes followed by several changes in distilled water was sufficient. If a secondary fixing solution was employed, the material was washed 5 hours or more in running water.

Method II. Bodian's No. 2 fixation was used with good results following either chlorethane or alcohol relaxation. Bodian's was made by using 90 cc. of 90 per cent alcohol, plus 4 cc. commercial formalin plus 5 cc. acetic acid. The fixing period was 4 days.

Method III. Zenker's fixative was employed in the Masson technique. The material remained in the Zenker's solution for 10 hours; then washed an equal length of time in running water.

Method IV. Helly's fluid, a modification of Zenker's, in which the acetic acid in the latter was replaced by 5 per cent formalin, and used with Mallory's phosphotungstic acid in combination with hemotoxylin.

DEHYDRATION, CLEARING, INFILTRATION AND EMBEDDING

Dehydration was accomplished with the aid of alcohol alone, or in combination with dioxane. In order to expedite these standardized procedures, the leech was cut into three equal lengths. When the central nervous system had previously been dissected such portions were carried intact through these steps. The above processes may, depending on the procedure followed, precede or follow the basic staining technique.

DEHYDRATION METHODS (PARAFFIN METHOD)

Method I. The gradual removal of water was facilitated by alcohol, proceeding in the usual routine of passing the material through ascending grades. From 100 per cent alcohol the material was passed into a mixture of equal parts of 100 per cent alcohol and zylol. This procedure was employed on mounted sections of small pieces, after the use of an aqueous stain. For mounted sections the time in each of the lower grades of alcohol was 3 minutes; for 100 per cent alcohol and for half alcohol and half xylene the time was 5 minutes each, except where otherwise noted.

Method II. This was one of several procedures followed for dehydration prior to paraffin embedding. The properly killed and fixed specimen, or selected portion, was placed in 70 per cent alcohol for 3 hours. The tissue was then placed for 2 hours each in 83-90-95 per cent alcohol, respectively.

Method III. A third method used to prepare material for paraffin infiltration was as follows: The specimen, or part thereof, was placed in 70 per cent alcohol for 3 hours. Two changes were made in this grade. The tissue was then transferred to 100 per cent dioxane for 3 hours.

DEHYDRATION METHOD (CELLOIDIN)

Method IV. In the dehydration of material for celloidin embedding, it was necessary to insure absolute removal of all water. The following procedure proved to be adequate for parts of the nervous system, or sections of the animal. Beginning with 30 per cent alcohol, the material was carried through 50-70-80-95-100 per cent for a minimum of 2 hours each. Two changes of 2 hours each in absolute alcohol-ether completed this step.

CLEARING METHODS

Clearing is the process of removing the alcohol from the section or specimen and the substitution of a fluid miscible with paraffin. In this step the tissue becomes translucent. Variations in method and material used in this step were dependent upon the nature of the tissue, and their place in the operation as a whole.

In preparing a specimen for infiltration and embedding in paraffin, the first two methods given below were satisfactory. It is my observation that for *H. marmoratis*, method II gives less hardening with a minimum of shrinkage.

Method I. Immediately following dehydration, the specimen was placed in a mixture of one-half butyl alcohol and one-half soft (48°) paraffin. The specimen remained in this mixture over night in a closed dish in an oven of 58° C. At the end of 12 to 14 hours, the specimen was ready for further infiltration. This method was employed after dehydration method III.

Method II. Dehydration by method II was followed by placing the specimen in a mixture of one-half chloroform and one-half soft paraffin. In this medium, the specimen was allowed to remain over night in a closed dish at room temperature. Further infiltration followed the regular procedure outlined below.

Method III. In the clearing of mounted sections following dehydration carbol xylene or xylene was used. The usual procedure of employing at least two changes of xylene was followed.

Method IV. Sections previously embedded in celloidin were cleared in cedar wood oil before mounting.

INFILTRATION AND EMBEDDING

Infiltration is the process of replacing the clearing agent with the material in which the tissue is to be imbedded. It also refers to the gradual impregnation of the tissue by celloidin, in instances where this latter is employed as the supporting medium. This step was accomplished by gradually infiltrating the tissue building up to the required density or concentration of the supporting agent.

Paraffin Method (Infiltration). Following clearing methods I or II, the material was placed in soft paraffin for two hours. This was replaced by a mixture of half soft and half hard (58°) paraffin and returned to the oven for two hours. The same procedure was repeated using hard paraffin. If used paraffin was employed, it was followed by an addition change of two hours in fresh paraffin. The hard paraffin, being the so-called "rubber paraffin," had a melting point of 56°–58° C. This same grade was used in the embedding process.

Paraffin Method (Embedding). This is a process in which the tissue or specimen is enclosed in a hardened block of supporting medium. Paraffin and celloidin are the conventional agents employed. Following the infiltration process, embedding is the final step prior to sectioning. In this step, as in all others, practice and repeated trials prove to be the best guide.

Plaster of Paris boxes were the most satisfactory embedding forms used for the paraffin method. Plaster of Paris boxes were soaked in water a few minutes prior to the introduction of the paraffin. The specimen was transferred from the infiltrating medium by warmed forceps, oriented, imbedding paraffin added, and a label inserted. As soon as a film forms over the surface of the paraffin the entire preparation was immersed in ice water, the contraction of the paraffin freeing the block. Many specimens can be so embedded in a short time, using not more than two plaster of Paris boxes in rotation.

Celloidin Method (Infiltration and Embedding). Material to be infiltrated and embedded in celloidin was dehydrated according to method IV. Following dehydration, the tissue was placed in 10 per cent celloidin for eight hours, then left over

night in 15 per cent celloidin. The material to be mounted was supported by a block of hardened celloidin, and oriented within a mould formed by surrounding the fiber cutting block with gummed paper. The cutting block mould was then filled with 15 per cent celloidin; the tissue oriented and hardened under a bell jar. Chloroform was used to complete the hardening. After a few hours the block was placed in thin cedar wood oil, where it remained until sectioned. It must be kept in mind for the above outlined procedure that the block of tissue was small.

The celloidin method of infiltration and embedding was the most satisfactory procedure where thick sections were desired. The celloidin method is particularly suitable for large tissue masses, but, in such instances infiltration and embedding may take days, or even weeks. It is essential that the tissue be infiltrated *gradually* and hardened slowly. Cedar wood oil was used in preference to alcohol during the cutting process. Sections were then cleared in thin cedar wood oil until further treatment.

STAINING METHODS

As previously stated, the writer was interested principally in behavior studies involving the neuro-anatomy of the leech. The application of neurological methods served only as a means toward the proposed objective. With all of the facilities of the Department of Anatomy of the Ohio State University at the writer's disposal, the initial project was begun. With the encouragement of the teaching staff and the able counsel of the Department's technician, many previously described methods were tried, none of which gave the desired results. It must not be inferred from this that the following tried and proven techniques failed entirely. On the contrary, thousands of sections proved extremely interesting. But in none of these, using the following basic procedures, were the desired elements sufficiently differentiated. The following are a few of the basic procedures first tried and later modified.

1. Activated protargol, D. Bodian.
2. Modified protargol, D. Bodian.
3. Formol thionin.
4. Methods I, II, III, and IV, (S. R. Cajal and deCastrol), Micro. Tech., McClung, 1937.
5. Iron hematoxylin.
6. Kopsch Golgi method.
7. N. C. Foot's modified Bielschowsky.
8. Ammoniated silver modification of J. W. Kernohan.
9. Masson technique.
10. Mallory's phosphotungstic acid and hematoxylin technique.
11. Ranson's pyridine silver method.

MILLER'S METHOD

The outlined procedure which follows was presented in detail by Miller (1944). This method combines several basic principles of neurological technique. Although it is a lengthy procedure it gives excellent differentiation to leech material. Sections prepared by this method are useful in both histological or cytological study.

The following outline denotes each step in the procedure:

1. Animal relaxed according to method I.
2. Killed and fixed as described in method I.
3. Nervous system dissected. A ventral strip of body wall may be left attached for convenience in orientation. A completely dissected nerve trunk was pinned on a strip of heavy paper bent to form a bow.
4. Tissue immersed in 3% potassium dichromate two weeks.
5. Rinsed in several changes of distilled water.
6. Tissue placed in 1% solution of silver nitrate; after precipitate forms, transfer to fresh 1% solution silver nitrate for one week.
7. Rinsed in several changes of distilled water.
8. Dehydrated, infiltrated and embedded as directed in preparation of celloidin sections.

9. Sections cut to desired thickness.
10. Sections transferred to thin cedar wood oil. This may be left here until clear, or until ready to proceed. Sections can be kept serially by separators.

At this point, two alternative procedures were followed—(A or B).

- 11A. Sections gradually hydrated and placed in
- 12A. Six per cent silver nitrate solution for four days (in dark).
- 13A. Washed in several changes of distilled water.
- 14A. Reduced for 24 hours in the following mixture (in dark):

Hydroquinone.....	2.0 g.
Neutral formalin.....	20.0 cc.
Water (distilled).....	200.0 cc.
- 15A. Washed in several changes of distilled water.
- 16A. (Optional) Tone in 0.2% gold chloride, 30 minutes.
- 17A. Washed in distilled water.
- 18A. Fixed in 5% hypo-sulphite of soda, 30 seconds.
- 19A. Washed in distilled water. (Counter stain at this point if desired.)
- 20A. Dehydrated, cleared, mounted in clarite.

OPTIONAL PROCEDURE:

- 11B. Sections from cedar wood oil were gradually hydrated then placed in
- 12B. 20% silver nitrate solution, 1 hour to 1½ hours.
- 13B. Washed quickly in distilled water and placed in
- 14B. Ammoniated silver nitrate, 3 minutes. This was prepared by adding concentrated aqua ammonia drop by drop to 20% silver nitrate solution until ppt. is almost dissolved and then filtered. This must be freshly prepared, and can be used but once.
- 15B. Washed quickly in distilled water.
- 16B. Sections placed in 10% formalin 1 minute.
- 17B. Washed in several changes of distilled water.
- 18B. Tone in 2.0% gold chloride, 10 minutes.
- 19B. Washed in distilled water.
- 20B. Optional procedure: Reduce in 2.0% oxalic acid plus 1 cc. formalin per 100 cc. of solution, 20 seconds.
- 21B. Washed in distilled water.
- 22B. Fixed in 5% hypo-sulphite of soda, 2 minutes.
- 23B. Washed in distilled water (counter stain at this point if desired).
- 24B. Dehydrated, cleared, mounted in clarite.

A counter stain was used successfully following either of the above variations. Mayer's acid hematoxylin and eosin, or Mayer's haemalum gave good cellular differentiation. Mallory's phosphotungstic acid hematoxylin as a counter stain was very effective following either of the above variations. Standard procedures were followed for counter staining and subsequent treatment.

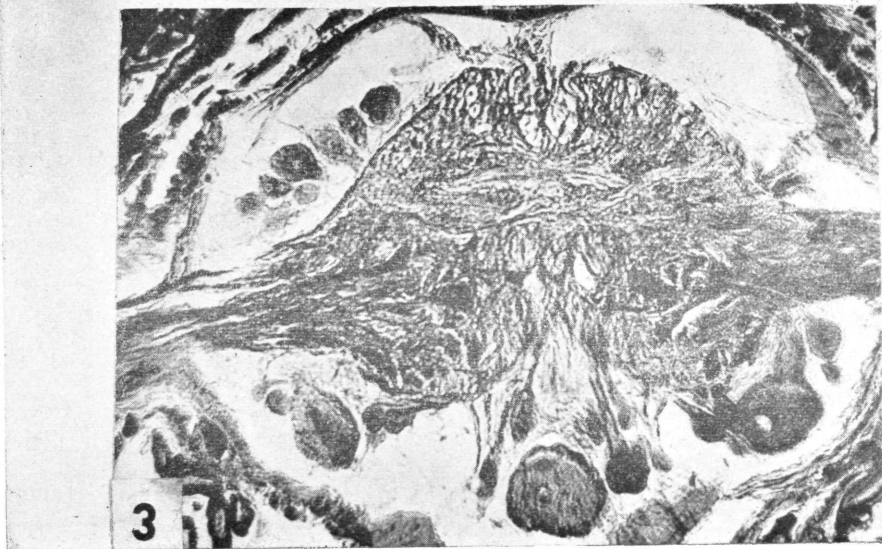
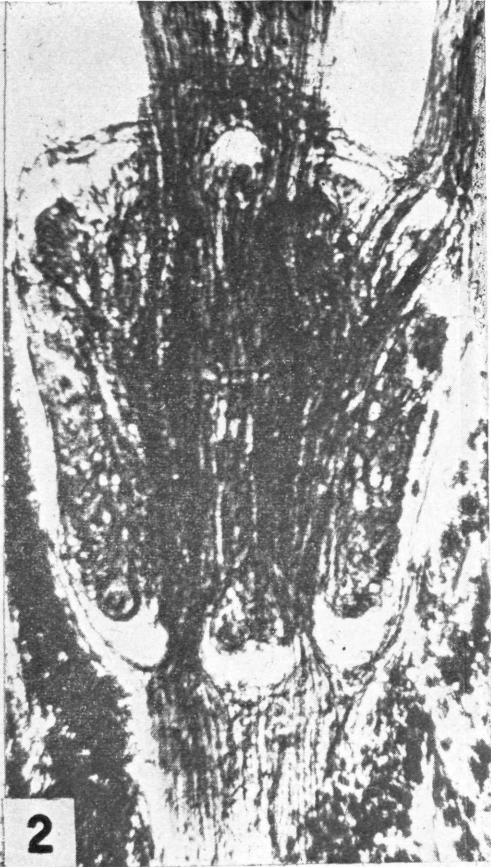
Method A produced the best material for histological study, particularly with reference to the longitudinal and frontal sections. Method B was especially useful in cytological study, being particularly valuable in sections through ganglia. Both variations were equally satisfactory for general morphological studies.

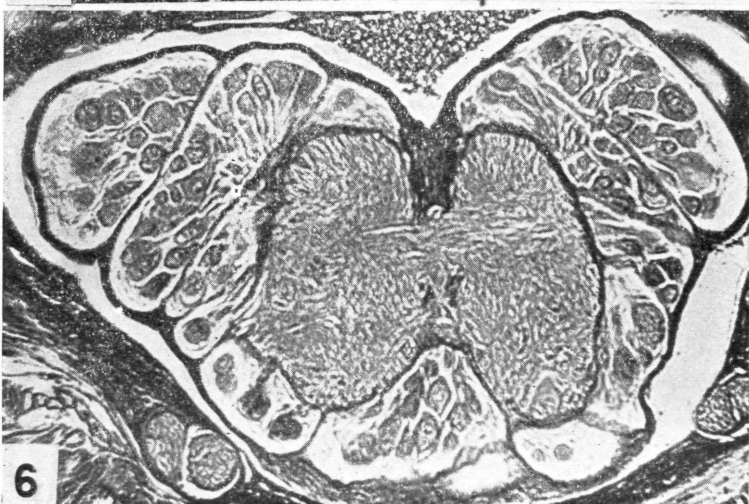
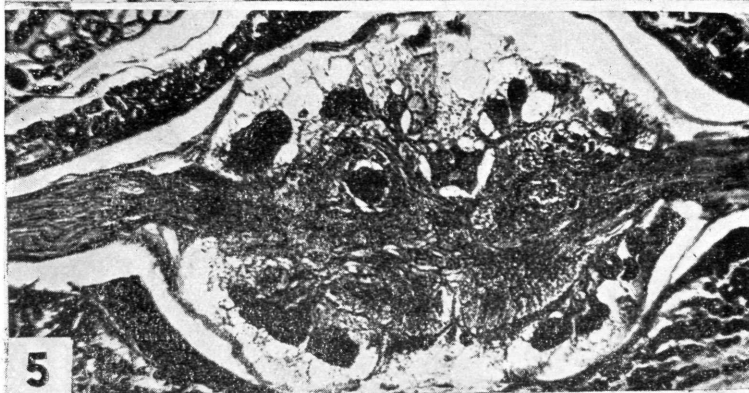
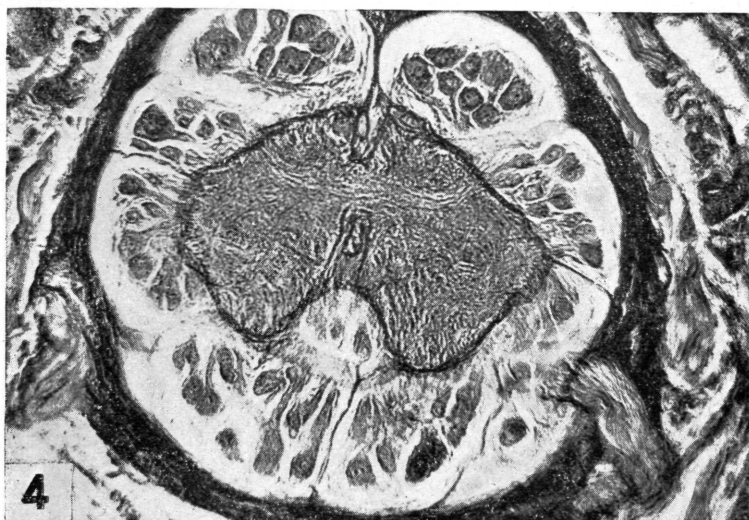
OTHER METHODS

In the course of this study many different techniques were employed. There were several which gave good supplementary results. In most instances, modifications from established procedures were necessary in adapting the technique to leech material. At this point the writer wishes to emphasize the importance of

EXPLANATION OF PLATE I

- FIG. 1. Photomicrograph—Method IV. Modification A. "Gross" nerve cell in supra-esophageal ganglion.
- FIG. 2. Photomicrograph—Miller's Method A. Frontal section through ganglion.
- FIG. 3. Photomicrograph—Method II. Cross-section through mid-body ganglion.





employing a variety of techniques when undertaking a comprehensive study. This necessitates the employment of diverse methods. In the study of no other tissue are these factors more important. Each new variation in technique supplements, rather than displaces, an established procedure. Each series adds its contribution to the total picture. The procedures which follow were found to be the most applicable, and the preparations the most usable.

I. Cajal-deCastro modification.

1. Relax animal according to method III.
2. Kill and fix in ammoniated absolute alcohol, 24 hours; *14 drops concentrated ammonia* to 100 cc. alcohol.
3. Dissect nervous system, then wash in 50% alcohol.
4. Place tissue in 6% silver nitrate, 72 hours, at 37° C., in dark.
5. Wash in distilled water.
6. Reduce 24 hours in dark in

10% neutral formalin.....	20 cc.
Hydroquinone.....	2 gm.
Distilled water.....	200 cc.
7. Wash in several changes of distilled water.
8. Tone in 0.2% gold chloride, 30 minutes.
9. Wash in several changes of distilled water.
10. Fix in 5% hyposulphite of soda, 30 seconds.
11. Wash in several changes of distilled water.
12. Dehydrate, as directed in method II.
13. Clear as in method II.
14. Infiltrate and embed by method I.
15. Section.
16. Clear as stated in method III.
17. Mount in clarite.

It will be noted that the amount of ammonia may be varied according to the specific nature of the nervous system used. Too much ammonia has a tendency to produce a faded out picture. It was noted that in sections containing both cerebral ganglia and ventral nerve cord, the two regions were not equally impregnated.

II. Bielschowsky modification.

1. Relax animal according to method I.
2. Kill and fix as described in method I, II, and IV. (Series employing each fixative.)
3. Dehydrate, method II, III.
4. Clear, method I and II.
5. Infiltrate and embed, method I.
6. Section, 10 μ .
7. Mount, remove paraffin, and gradually hydrate.
8. Wash in distilled water.
9. Place slides in 20% silver nitrate, one hour at room temperature.
10. Wash quickly in distilled water (two dips in each of two changes) and place in
11. Ammoniated silver nitrate prepared as in 14B, Miller's Method.
12. Wash quickly in distilled water.
13. Sections to 10% formalin, 30 seconds.
14. Wash in distilled water; several changes.
15. Tone in 0.2% gold chloride, 20 minutes.
16. Wash in distilled water.
17. Reduce in 2.0 oxalic acid, plus 1 cc. of 40% formalin per 100 cc. of solution, 30 seconds.
18. Wash in distilled water.
19. Fix in 2.0% hyposulphite of soda, 2 minutes.
20. Wash in several changes of distilled water.
21. Dehydrate, method I.
22. Clear, method III.
23. Mount in clarite.

EXPLANATION OF PLATE II

- FIG. 4. Photomicrograph—Method III. Cross-section through caudal ganglion.
 FIG. 5. Photomicrograph—Method I. Cross-section through mid-body ganglion.
 FIG. 6. Photomicrograph—Method III. Cross-section through subesophageal ganglion.

III. Masson stain.

This standard stain technique was used with variations as indicated in the following:

1. Relaxing, method I.
2. Killing and fixing, method II and III.
3. Dehydration, method II.
4. Clearing, method II.
5. Infiltration and embedding, method I.
6. Section, mount and remove paraffin.
7. Gradual hydration, stain 5 minutes in Mayer's haemalum.
8. Rinse in distilled water, wash in tap water until blue, and return to distilled water.
9. Stain 5 minutes in

2 pints ponceau de xylidine 1%	In 1% acetic acid in
1 pint Acid fuchsin	distilled water.
10. Rinse in distilled water, 2 changes.
11. Mordant 5 minutes in 5% phosphotungstic acid.
12. Rinse in distilled water.
13. Stain 5 minutes in 2% aniline blue in 2% acetic acid.
14. Rinse in distilled water.
15. Sections to 1% acetic acid, 5 minutes.
16. Sections transferred to distilled water, then
17. Direct to 95% alcohol. Differentiate in 95%. Several changes; if stain is light pass through quickly. If blue has overstained, place slides in 70% alcohol overnight and restain.
18. Five minutes in half absolute and half xylene.
19. Xylene; two changes, each 5 minutes.
20. Mount in clarite.

It will be noted that there are several minor changes from the regular routine, particularly with reference to the killing and fixing procedure. This stain gave excellent cellular and tissue differentiation.

IV. Mallory's phosphotungstic acid hematoxylin method.

Modifications in this technique were largely confined to variations in killing and fixing procedure. This method provided a valuable series of sections illustrating not only the intra-cellular fibrils, but cytological details of ganglion cells. Mallory and Parker in McClung have described the preparation of the stain and certain prescribed steps. Leech material was prepared by the writer using the following combinations of relaxing, killing, and fixing methods followed by paraffin embedding.

- 1A. Relax by method I, kill and fix, method I.
- 2A. Dehydrate by method III, clear, method I, embed, method I.
- 1B. Relax by method III, kill and fix, method II.
- 2B. Dehydrate, method II, clear, method II, embed, method I.
- 1C. Relax by method I, kill and fix, method IV.
- 2C. Dehydrate, method II, clear, method II, embed, method I.

For the convenience of the reader, the following condensed outline of the routine procedure as given by McClung follows:

1. Sections hydrated (if Helly's fixative was used, treat sections in 70% to which has been added iodine) 0.4% iodine in 70% alcohol, 3 minutes. Bleach and wash in 0.25% sodium hyposulphate, 3 minutes. Wash in 70% or 50%, 5 minutes. Sections passed through 35% to distilled water.
2. Sections to 0.25% aqueous solution of potassium permanganate, 10 minutes.
3. Wash in several changes of distilled water.
4. Sections to 5% oxalic acid, 20 minutes.
5. Wash thoroughly in several changes of distilled water.
6. Stain 20-24 hours in Mallory's phosphotungstic acid, hematoxylin.
7. Sections direct to 95% alcohol (quick).
8. Sections to absolute (quick).
9. Sections to absolute and xylene, 50-50.
10. Sections to xylene, 2 changes.
11. Mount in clarite.

It will be noted that this procedure follows the recommendations of Mallory, with the exception of the killing and fixing agents employed, and the special treatment following Helly's fixative.

V. Formal thionin technique as applied to the leech.

1. Relax by method II.
2. Kill, fix and stain, 26 days in the following:
 .25% thionin.
 10% formalin.
3. Dehydrate in dioxane, 3 changes of 3 hours each.
4. Clear by method I.
5. Infiltrate and embed by method I.
6. Section, mount, remove paraffin in two changes of xylene (five minutes), add clarite and cover slip.

The sections so prepared are usable in general morphological study. It is not intended the thionin be presented as a neurological stain. It produces, however, a pleasing picture of the general morphology of the tissue, and is extremely useful in orientation.

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